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# Extended Multilocus Sequence Typing System for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*

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**A multilocus sequence typing (MLST) system has been reported previously for *Campylobacter jejuni* to both differentiate strains and identify clonal lineages. However, sequence variation at the MLST loci prevents its use for closely related *Campylobacter* species. We describe herein an expanded MLST method to include three clinically relevant *Campylobacter* species, *C. coli*, *C. lari*, and *C. upsaliensis*, and a fourth *Campylobacter* species, *C. helveticus*. The *C. coli* and *C. helveticus* methods use the same seven *C. jejuni* loci (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*); however, *adk* and *pgi* were substituted for *aspA* and *gltA* in *C. lari* and for *gltA* and *pgm* in *C. upsaliensis*. Multiple *C. coli* ($n = 57$), *C. lari* ($n = 20$), *C. upsaliensis* ($n = 78$), and *C. helveticus* ($n = 9$) isolates, representing both clinical and environmental sources, were typed. All four species were genetically diverse: the majority (>80%) of the isolates had unique sequence types (STs). Using this method, mixed *C. lari*, *C. upsaliensis*, and *C. helveticus* isolates were identified; upon separation, each isolate was shown to contain two strains of the same species with distinct STs. Additionally, the expanded MLST method was able to detect potential lateral transfer events between *C. jejuni* and either *C. coli* or *C. lari* and between *C. upsaliensis* and *C. helveticus*. Thus, the expanded MLST method will prove useful in differentiating strains of five *Campylobacter* species, identifying mixed *Campylobacter* cultures, and detecting genetic exchange within the genus.**

*Campylobacter* spp. are a major cause of human bacterial gastrointestinal illness in the developed world (1, 9, 23, 24, 61). The incidence of reported campylobacteriosis in the United States in 2003 was 12.6 cases per 100,000 persons, second only to infections by *Salmonella* spp. (14.5 cases per 100,000 persons) (9). The majority of campylobacterioses are caused by *Campylobacter jejuni*; however, the causative agents in many of these illnesses are typed only to the genus level, i.e., *Campylobacter* spp. *C. jejuni* is highly prevalent in poultry (4, 5, 19, 31), and poultry products are often assumed to be the source of most *C. jejuni* infections (22, 30, 49, 58, 62), although poultry isolates may not all be equally pathogenic (25, 51). Other sources, such as untreated water (7, 15, 29) and unpasteurized milk (18, 34), can also lead to campylobacteriosis. *C. jejuni* infections are mainly sporadic, although occasional outbreaks can occur (23, 54). The necessity of a reliable typing method to characterize *C. jejuni* strains and investigate the epidemiology of *C. jejuni* infections provided the impetus for the development of a multilocus sequence typing (MLST) system for *C. jejuni*. This method was developed by Dingle et al. (12) and has been used successfully to characterize *C. jejuni* strains (11, 13, 43, 59, 63) and investigate *C. jejuni* outbreaks (60). As with MLST methods developed in other taxa, this system amplifies and sequences portions of seven housekeeping genes. Based on the sequence information at each locus, allele numbers are assigned, with distinct allele sequences receiving arbitrary allele numbers. Typing of 194 strains identified 155 sequence types (STs); each ST consists of a unique allelic profile. *C. jejuni* allele sequences and sequence types were made available in a web-based *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). Since the development of this typing scheme, >1,000 STs have been identified. Selection of the seven housekeeping genes was based on the ability to amplify these genes from a diverse group of sources, sufficient sequence variation at each locus, and the absence of positive selection for each locus (12). The seven housekeeping genes chosen were *aspA* (aspartase A), *atpA* (ATP synthase α subunit; termed *uncA* in reference 12), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (actually *Cj0360*; phosphoglucomutase), and *tkt* (transketolase).

Although the majority of *Campylobacter* infections are caused by *C. jejuni*, other *Campylobacter* species, e.g., *C. coli*, *C. lari*, and *C. upsaliensis*, have been associated with either sporadic (10, 28, 37, 39, 52, 55, 65) or outbreak (8, 26, 40, 57) cases of gastroenteritis in humans. *C. jejuni* and *C. coli* are often isolated from the same hosts (48). *C. lari* and *C. upsaliensis* are infrequent contaminants of poultry (2, 42), but *C. lari* has been isolated from shellfish (i.e., mussels and oysters) (14, 66). *C. upsaliensis* is predominantly associated with domestic dogs and cats (3, 17); a related *Campylobacter* sp., *C. helveticus*, has also been isolated from dogs and cats (64) but has not been shown to cause human illness.

The current MLST method is designed to type only *C. jejuni* strains. Because of the nondegenerate nature of the *C. jejuni* MLST primer sets and substantial sequence diversity between

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*C. jejuni* and other *Campylobacter* species (21), these primer sets can be used to type some *C. coli* alleles or loci but cannot be used to type other *Campylobacter* species (e.g., *C. lari* and *C. upsaliensis*). Therefore, although the *C. jejuni* MLST method has identified potential genetic exchange between *C. jejuni* and *C. coli* (45, 63), it cannot identify genetic exchange and recombination between other *Campylobacter* species; such genetic exchange has been proposed to play a role in the evolution of the genus (45, 46). Thus, there is a need for an extended MLST scheme that types the non-*C. jejuni* campylobacters. The recent draft sequencing of the genomes of three additional *Campylobacter* species (i.e., *C. coli*, *C. upsaliensis*, and *C. lari* [21]) was available to expand the current *C. jejuni* MLST system to include these thermotolerant and clinically relevant *Campylobacter* species.

Here, we describe an expanded MLST method for *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. The genomic sequences for these strains were used to construct novel, degenerate primer sets which can amplify seven housekeeping loci from five *Campylobacter* species (including *C. jejuni*). Over 150 *Campylobacter* strains, isolated from both clinical and environmental sources, were characterized with this system. One hundred twenty-eight STs were identified as well as clonal lineages in each species. Additional advantages of this MLST method were the capabilities of detecting mixed cultures of *Campylobacter* and genetic exchange and recombination between *Campylobacter* species.

## MATERIALS AND METHODS

**Growth conditions and chemicals.** All *Campylobacter* strains were cultured routinely at 37°C on brucella agar amended with 5% (vol/vol) laked horse blood (BAB) (Hema Resource & Supply, Aurora, Oreg.). Atmospheric conditions for all strains were 5% $H_2$, 10% $CO_2$, and 85% $N_2$. PCR enzymes and reagents were purchased from New England Biolabs (Beverly, Mass.) or Epicentre (Madison, Wis.). All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, Mo.) or Fisher Scientific (Pittsburgh, Pa.). DNA sequencing chemicals and capillaries were purchased from Applied Biosystems (Foster City, Calif.).

**DNA purification, amplification, and sequencing.** *Campylobacter* genomic DNA was prepared as follows: cells were scraped from agar plates and resuspended in 1.5 ml 10% (wt/vol) sucrose, 50 mM Tris (pH 8.0). Two hundred fifty μl of a 10-mg $ml^{-1}$ lysozyme solution (in 250 mM Tris, pH 8.0) and 600 μl of 0.1 M EDTA were then added to the suspension. The suspension was incubated for 10 min on ice, then 300 μl of a 5% (wt/vol) sodium dodecyl sulfate solution was added, and the mixture was vortexed briefly to clarify the solution. The lysates were incubated sequentially with 25 μl RNase A (1 mg $ml^{-1}$) and 10 μl proteinase K (10 mg $ml^{-1}$), and the DNA was spooled following addition of sodium acetate (1/10 volume) and ethanol (room temperature, 2 volumes). DNA was resuspended in Tris-EDTA (pH 8.0), extracted twice with phenol-chloroform (1:1, vol/vol) and once with chloroform, and concentrated by ethanol precipitation.

PCRs were performed on an MJ Research (South San Francisco, Calif.) Tetrad thermocycler with the following settings: 30 s at 94°C, 30 s at 53°C, and 2 min at 72°C (30 cycles). Each amplification mixture contained 50 ng genomic DNA, 1× PCR buffer (Epicentre), 1× PCR enhancer (Epicentre), 2.5 mM $MgCl_2$, 250 μM (each) deoxynucleoside triphosphates, 50 pmol each primer, and 0.2 U polymerase (New England Biolabs). Amplicons were purified on a BioRobot 8000 workstation (Qiagen, Santa Clarita, Calif.).

Cycle sequencing reactions were performed on an MJ Research Tetrad thermocycler using the ABI PRISM BigDye terminator cycle sequencing kit (version 3.0) and standard protocols. All extension products were purified on DyeEx 96-well plates (Qiagen). DNA sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using the POP-6 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software. PCR/sequencing oligonucleotides were purchased from Qiagen.

**Separation of mixed cultures.** Strains *C. coli* RM1908, *C. lari* RM2816, *C. upsaliensis* RM3949, *C. upsaliensis* RM4048, and *C. helveticus* RM4087 were grown for 48 h on BAB plates. Approximately 5 to 10 μl of cells was removed from the plates with 1-μl loops and resuspended in 1 ml phosphate-buffered saline (pH 7.0). The suspensions were sonicated (VWR Ultrasonic Bath Sonicator model 75T; VWR, West Chester, Pa.) for 4 min at the default intensity, vortexed for 30 s, dilution plated onto fresh BAB plates, and incubated for 48 h. For each strain, 8 or 16 well-isolated colonies were picked, resuspended in 100 μl 10 mM Tris (pH 8.0), lysed in a thermocycler (5 min at 94°C), and centrifuged to pellet cell debris. As a quick screen to determine which colonies represented different sequence types, genomic DNAs from all cell lysates were first amplified and sequenced using the aspAF1/aspAR1 (for strain RM1908) or aspAF2/aspAR2 (for strains RM2816, RM3949, RM4048, and RM4087) primer set. *aspA* allele sequences for each strain were aligned, and genomic DNAs representing different alleles were identified. These genomic DNAs were amplified subsequently and sequenced using all seven MLST primer sets. In both rounds of amplification, reaction conditions were as described above using 2 μl lysed culture supernatant per reaction.

**Assignment of allele numbers, sequence types, and clonal complexes.** The Perl program MLSTparser was written to extract allele sequences and assign allele numbers and sequence types. With an input of FASTA-formatted files representing the forward and reverse reads for each isolate, MLSTparser extracts the in-frame internal gene fragments from each read and compares the fragment sequence from the forward read and the complemented fragment sequence from the reverse read. Forward and complemented reverse sequences that are not identical are not analyzed further. MLSTparser then assigns allele numbers arbitrarily to unique sequences for each locus in the order that they are identified, by increasing "RM" strain number in this study. Similarly, sequence types are assigned arbitrarily to unique allelic profiles. All allelic sequences were queried against the *Campylobacter jejuni/coli* MLST database (http://pubmlst.org/campylobacter/) and assigned secondary allele numbers, where applicable.

Sequence types were grouped into clonal complexes using the program eBURST (http://eburst.mlst.net) (20). Clonal complexes were defined as groups of two or more independent isolates that shared identical alleles at five or more loci. Where applicable, each complex was named after the putative founder sequence type (e.g., ST-1 complex). Unweighted pair group method with arithmetic mean (UPGMA) dendrogram construction and calculation of the $d_n/d_s$ ratios were performed using the computer program START (33). Variable sites were identified using MEGA version 2.1 (36).

**AFLP profiling of *C. upsaliensis* strains.** Genomic DNA was extracted from strains by use of an Easy-DNA kit (Invitrogen, Carlsbad, CA; protocol 3 per the manufacturer's instructions). AFLP profiling was performed subsequently by use of the method described by Siemer et al. (63a). Briefly, approximately 625 ng genomic DNA was digested with 1 U MfeI and 1 U BspDI in NEB4 buffer (New England Biolabs) for 1 h at 37°C, and adaptor sequences complementary to the restriction sites were ligated to the restriction fragments by the addition of 1 U T4 DNA ligase, 2 μl 10× T4 DNA ligase buffer (USB Corporation, Cleveland, Ohio), 2 μM FC adaptor, and 20 μM RC adaptor. After a 3-h incubation period at 37°C, the reaction mixture was diluted (1:25 ratio) with sterile, double-distilled water. PCR was then performed by use of nonselective, half-site-specific primers MfeI-F (5′ GAG AGC TCT TGG AAT TG 3′, FAM [6-carboxylfluorescein] labeled at the 5′ end) and BspDI (5′ GTG TAC TCT AGT CCG AT 3′) (DNA Technology, Århus, Denmark). Amplification conditions were as described previously (35), except that a 25-cycle program was used. AFLP fragments were detected on an ABI 377 automated sequencing machine (Applied Biosystems, Foster City, Calif.), and data were collected and analyzed with GeneScan v. 3.1 (Applied Biosystems, Foster City, Calif.) and BioNumerics v. 3.0 (Applied Maths, Kortrijk, Belgium), as described previously (53).

## RESULTS

**Experimental rationale.** A multilocus sequence typing system for *C. jejuni* was described recently by Dingle et al. (12). The recent draft sequencing of the genomes of three additional *Campylobacter* species (i.e., *C. coli*, *C. upsaliensis*, and *C. lari* [21]) permitted an expansion of the *C. jejuni* MLST system to include these additional species. To keep the expanded MLST system as analogous as possible to the system described by Dingle et al. (12), we wanted our new system to include the same seven housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*,

TABLE 1. *Campylobacter* expanded MLST primer sets

| Locus | Oligonucleotide primer set | | | | Amplification[a] | | | | | Amplicon size (bp) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Forward (5′–3′) | | Reverse (5′–3′) | | Cj | Cc | Cl | Cu | Ch | |
| | Primer | Sequence | Primer | Sequence | | | | | | |
| *adk* | adkF | TGAAAGAATTRTTTTTAATCATAGG | adkR | CTTTCATRTCWGCHACGATAGGTTC | Y | Y | **Y** | **Y** | Y | 545–546 |
| *asp* | aspAF1 | GAGAGAAAAGCWGAAGAATTTAAAGAT | aspAR1 | TTTTTTCATTWGCRSTAATACCATC | Y | **Y** | NT | NT | NT | 676 |
| | aspAF2 | GAAGCWAAAGCWAAAGAATAYAAAGAT | aspAR2 | GAGTTTTTTGCAWGCTTCWGGATT | NT | NT | N | **Y** | **Y** | 690 |
| *atpA* | atpAF | GWCAAGGDGTTATYTGTATWTATGTTGC | atpAR | TTTAADAVYTCAACCATTCTTTGTCC | Y | **Y** | **Y** | **Y** | **Y** | 700 |
| *glnA* | glnAF | TGATAGGMACTTGGCAYCATATYAC | glnAR | ARRCTCATATGMACATGCATACCA | Y | **Y** | **Y** | **Y** | **Y** | 751 |
| *gltA* | gltAF | GARTGGCTTGCKGAAAAYAARCTTT | gltAR | TATAAACCCTATGYCCAAAGCCCAT | Y | **Y** | N | N | **Y** | 706 |
| *glyA* | glyAF | ATTCAGGTTCTCAAGCTAATCAAGG | glyAR | GCTAAATCYGCATCTTTKCCRCTAAA | Y | **Y** | **Y** | **Y** | **Y** | 716 |
| *pgi* | pgiF1 | TAGTGGGWATGGGAGGDTCAAGTT | pgiR1 | CCAATDAGWGCDATAGGAGTTAAACC | Y | Y | **Y** | Y | N | 646–649 |
| | pgiF2 | TTTAGTGGGWATGGGTGGKTCAAGT | pgiR2 | GCAATAGGAGTTAAACCTATRCGTT | NT | Y | NT | NT | N | 642 |
| | pgiF2 | TTTAGTGGGWATGGGTGGKTCAAGT | pgiR3 | TCTCTAGCACCAATGAGAGCTATGG | NT | NT | NT | **Y** | N | 660 |
| *pgm* | pgmF1 | CATTGCGTGTDGTTTTAGATGTVGC | pgmR1 | AATTTTCHGTBCCAGAATAGCGAAA | Y | **Y** | N | N | N | 720 |
| | pgmF2 | ATGTGGCWCAYGGAGCRGCTTATAA | pgmR2 | GGCTATTRATRCCCTTTTTATCAAG | NT | NT | NT | Y | **Y** | 675 |
| | pgmF3 | CGTGTTGTTTTAGATGTGGCTCA | pgmR3 | ATAGCGAAACAAACTAGCAATTCCT | NT | NT | **Y** | NT | NT | 699 |
| *tkt* | tktF1 | GCAAAYTCAGGMCAYCCAGGTGC | tktR | TTTTAATHAVHTCTTCRCCCAAAGGT | Y | **Y** | N | **Y** | **Y** | 730 |
| | tktF2 | GCCTTTGGGTTTAGCRGATATTATG | tktR | TTTTAATHAVHTCTTCRCCCAAAGGT | NT | NT | **Y** | NT | N | 706 |

[a] Cj, *Campylobacter jejuni*; Cc, *Campylobacter coli*; Cl, *Campylobacter lari*; Cu, *Campylobacter upsaliensis*; Ch, *Campylobacter helveticus*; NT, not tested; Y, all isolates amplified; N, at least one isolate did not amplify. **Y**, primers were used in the final MLST schemes.

*pgm*, and *tkt*) and the same in-frame internal fragments. Additionally, in order to minimize the number of oligonucleotide primers required by the expanded system, each primer set should amplify as many species as possible, preferably all four (including *C. jejuni*). Our criteria for the expanded *Campylobacter* MLST system included both versatility and discriminatory power. Primer sets that did not amplify every isolate for a given species, or loci in which most or all alleles were identical, would be excluded from the final system for that species.

**Design and characterization of the extended *Campylobacter* multilocus sequence typing system.** To design a universal set of *Campylobacter* MLST primers, the sequences of the seven housekeeping genes described above were extracted from the complete or draft sequences of *C. jejuni* strains NCTC 11168 and RM1221, *C. coli* strain RM2228, *C. upsaliensis* strain RM3195, and *C. lari* strain RM2100 and aligned. The sequences of two additional housekeeping genes, *adk* (adenylate kinase) and *pgi* (glucose-6-phosphate isomerase), used previously in a second *C. jejuni* MLST method (43), were also extracted and aligned. Such alignments would be beneficial in the construction of universal primer sets; presumably, degenerate primers designed to amplify all four species would also amplify most, if not all, of the strains of each species. Despite substantial nucleotide sequence diversity between all four species, degenerate primer sets (Table 1) that would amplify genomic DNA from all four *Campylobacter* species could be constructed for four loci (*adk*, *atpA*, *glnA*, and *glyA*). Surprisingly, these same primer sets also amplified genomic DNA from isolates of a fifth species, *C. helveticus*, suggesting that the new MLST scheme could be expanded to type five *Campylobacter* species. Unfortunately, analysis of the closed *C. lari* RM2100 genome and PCR analysis of 20 other *C. lari* strains indicated that the gene encoding citrate synthase, *gltA*, was absent in RM2100 and possibly absent in *C. lari* in general. Sequence diversity at the remaining loci prevented the construction of single, universal primer sets: two different primer sets were constructed for the *aspA* and *tkt* loci and three primer sets were constructed for the *pgi* and *pgm* loci (Table 1).

To test these primer sets, genomic DNA from 57 *C. coli*, 20 *C. lari* (including four urease-positive strains), 78 *C. upsaliensis*, and 9 *C. helveticus* strains was amplified and sequenced. Every *C. coli* strain amplified with primer sets for all nine loci; however, to keep the *C. coli* and *C. jejuni* MLST methods comparable, the final seven loci were the same as described for *C. jejuni*. In contrast, either the loci of several *C. lari*, *C. upsaliensis*, or *C. helveticus* strains were inconsistently amplified (*C. lari aspA*, *C. upsaliensis gltA*, and *C. helveticus pgi*) or the resulting alleles were insufficiently variable (*C. lari aspA* and *C. helveticus adk*). Therefore, these loci were not included in the final expanded typing systems. The final *C. upsaliensis* typing system includes the following seven loci: *adk*, *aspA*, *atpA*, *glnA*, *glyA*, *pgi*, and *tkt*. The *C. lari* MLST system contains the following seven loci: *adk*, *atpA*, *glnA*, *glyA*, *pgi*, *pgm*, and *tkt*. The final *C. helveticus* typing system includes the same seven loci as described for *C. jejuni*.

**Genetic diversity at the expanded *Campylobacter* MLST loci.** Although a limited number of *C. lari* and *C. helveticus* isolates were typed, many alleles were found at each of the nine loci in these two species (Table 2): all eight of the *C. helveticus* isolates contained different *glyA* alleles and 14 of 19 (73.7%) *C. lari* isolates contained different *adk* alleles. A large percentage of variable sites were found in *C. lari* (15.5% to 19.1%). Many of these variable sites were due to the presence of five strains in the sample set: the urease-positive isolates RM3659, RM3660, RM3661, and RM4110 and the divergent isolate RM2824. The ratio of nonsynonymous to synonymous base

TABLE 2. Diversity at the expanded *Campylobacter* MLST loci

| Locus | Species | Alleles (% of isolates)[a] | Variable sites (%)[b] | $d_n/d_s$[c] |
|---|---|---|---|---|
| *adk* | *C. lari* | 14 (73.7) | 72 (19.1) | 0.047 |
| | *C. upsaliensis* | 21 (27.6) | 25 (6.6) | 0.097 |
| *aspA* | *C. coli* | 5 (8.9) | 5 (1.1)[d] | 0.000 |
| | *C. upsaliensis* | 33 (43.4) | 57 (11.9) | 0.043 |
| | *C. helveticus* | 7 (87.5) | 20 (4.2) | 0.161 |
| *atpA* | *C. coli* | 4 (7.1) | 6 (1.2) | 0.000 |
| | *C. lari* | 12 (63.2) | 76 (15.5) | 0.000 |
| | *C. upsaliensis* | 20 (26.3) | 31 (6.3) | 0.008 |
| | *C. helveticus* | 5 (62.5) | 9 (1.8) | 0.000 |
| *glnA* | *C. coli* | 5 (8.9) | 4 (0.8) | 0.000 |
| | *C. lari* | 9 (47.4) | 78 (16.4) | 0.000 |
| | *C. upsaliensis* | 26 (34.2) | 22 (4.6) | 0.048 |
| | *C. helveticus* | 5 (62.5) | 26 (5.5) | 0.000 |
| *gltA* | *C. coli* | 6 (10.7) | 5 (1.2) | 0.000 |
| | *C. helveticus* | 4 (50) | 4 (1.0) | 0.580 |
| *glyA* | *C. coli* | 11 (19.6) | 10 (2.0) | 0.000 |
| | *C. lari* | 8 (42.1) | 86 (17.0) | 0.035 |
| | *C. upsaliensis* | 28 (36.8) | 34 (6.7) | 0.010 |
| | *C. helveticus* | 8 (100) | 25 (4.9) | 0.284 |
| *pgi* | *C. upsaliensis* | 24 (31.6) | 28 (6.1) | 0.070 |
| *pgm* | *C. coli* | 7 (12.5) | 16 (3.2) | 0.145 |
| | *C. lari* | 11 (57.9) | 77 (15.5) | 0.024 |
| | *C. helveticus* | 5 (62.5) | 80 (16.1) | 0.020 |
| *tkt* | *C. coli* | 8 (14.3) | 25 (5.4) | 0.173 |
| | *C. lari* | 11 (57.9) | 74 (16.1) | 0.035 |
| | *C. upsaliensis* | 28 (36.8) | 39 (8.5) | 0.045 |
| | *C. helveticus* | 2 (25) | 2 (0.4) | 0.281 |

[a] % of isolates = number of alleles/strains typed.
[b] % variable sites = polymorphic sites/allele size (nucleotides).
[c] Ratio of nonsynonymous to synonymous sites.
[d] Does not include 2225*aspA*. Variable sites with 2225*aspA* are 56 (11.7%).

substitutions ($d_n/d_s$) ranged from 0 to 0.173 for *C. coli*, 0 to 0.047 for *C. lari*, 0.008 to 0.097 for *C. upsaliensis*, and 0 to 0.580 for *C. helveticus* (Table 2). With three exceptions (*C. helveticus gltA*, *glyA*, and *tkt*), these ratios were much less than 1; it is unclear what the high values for the *C. helveticus gltA*, *glyA*, and *tkt* loci represent.

**Sequence types and clonal complexes.** In accordance with the large number of alleles present in the four species at each of the nine loci, 126 STs were identified among the 152 isolates typed in this study: 37 STs in *C. coli*, 15 STs in *C. lari*, 66 STs in *C. upsaliensis*, and 8 STs in *C. helveticus* (Tables 3 to 6). Many of these STs were unique in the sample set, the most commonly identified STs being *C. coli* ST-1058 ($ST_C$-1058) and *C. upsaliensis* ST-12 ($ST_U$-12). The majority (31 of 37, 83.8%) of the *C. coli* STs were assigned to the ST-1017 complex; a smaller clonal complex, ST-1052, contained four members and two STs. One complex, termed ST-2, was present in *C. lari*. The *C. lari* ST-2 complex had five members but only two STs, $ST_L$-2 and $ST_L$-6. Six complexes were identified in *C. upsaliensis*. The ST-42 complex was the largest, with 20 members and 14 STs. The five other complexes, termed ST-16, ST-35, ST-45, ST-50, and ST-64, contained between two and four STs. Only one complex was identified in *C. helveticus*: ST-1 with two member STs.

In *C. coli* and *C. lari*, no correlation could be made between membership in any of the three complexes and the isolate source, the geographic location in which the strain was isolated, or the date of isolation. No definitive genogroups were identified by UPGMA analysis of the *C. coli* allele profiles (Fig. 1) However, three clonal complexes in *C. upsaliensis* (ST-42, ST-45, and ST-50) showed a definitive correlation with both isolate source and geographic location. Members of the ST-42 complex were all clinical isolates from South Africa, and members of both the ST-45 and ST-50 complexes were all clinical isolates from either Belgium or France (Table 5). Additionally, some isolates from household pets were associated with minor clonal complexes. In contrast, canine or clinical *C. upsaliensis* strains isolated at the California Department of Health Services laboratory in Los Angeles, CA (37), were all assigned unique STs; three of the STs ($ST_U$-19, $ST_U$-25, and $ST_U$-27) originated from the same household (Table 5). UPGMA analysis of the *C. upsaliensis* allele profiles defines two genogroups (Fig. 2). Genogroup II contains four subgroups, termed here "A" through "D." Phylogenetic analysis of each of the seven loci produces dendrograms with similar topologies (data not shown), indicating that the phenogram in Fig. 2 is an accurate representation of genetic differences between the strains. Genogroups IIA and IIB are comprised exclusively of *C. upsaliensis* isolates from Belgium and France, genogroup IIC is comprised exclusively of isolates from South Africa, and genogroup IID is comprised of isolates from Belgium, South Africa, and the United Kingdom.

**Comparison of *C. upsaliensis* MLST and AFLP profiles.** Reproducibility of the AFLP method was evaluated by examining nine *C. upsaliensis* strains in duplicate experiments. Strains were examined between two and four times on different occasions, and a total of 21 duplicate profiles were used. The mean similarity between paired, duplicate strain profiles was determined as 91%. The *C. upsaliensis* strains studied gave unique AFLP profiles containing 9 to 29 fluorescently labeled fragments (Fig. 3). Results of the cluster analysis showed an excellent correlation with that of the MLST data. Three major clusters were formed at the 50% similarity (S level), of which all strains in AFLP cluster 1 appeared phylogenetically related in the MLST analysis (Fig. 2). AFLP cluster 2 strains were similarly assigned to MLST cluster II (Fig. 2), with representatives of MLST subphenons IID and IIC, respectively, sharing a higher level of similarity by AFLP analysis to other representatives of the same subphenon. Moreover, the only two strains belonging to MLST subphenon IIC assigned to the same sequence type complex (RM3776 and RM3779; ST-42 complex) were highly related by AFLP analysis (85% similar). AFLP cluster 3 contained the only member of MLST cluster IIB studied.

**Analysis of mixed *Campylobacter* isolates.** During the course of sequencing the MLST amplicons, we noticed that traces from several, but not all, loci in five strains (*C. coli* strain RM1908, *C. lari* strain RM2816, *C. upsaliensis* strains RM3949 and RM4048, and *C. helveticus* strain RM4087) contained both a primary and secondary peak at certain nucleotide positions. These loci were reamplified and resequenced with identical results. Comparison of the forward traces with the reverse-

TABLE 3. Allele numbers, sequence types, and lineages for *C. coli* isolates ($n = 56$)[a]

| Lineage | ST | Allele | | | | | | | Isolate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *aspA* | *atpA* | *glnA* | *gltA* | *glyA* | *pgm* | *tkt* | Name | Source | Yr | Location |
| ST-1017 complex | 825 | 33 | 17 | 39 | 30 | 82 | 113 | 47 | RM1534 (ATCC 49941) | Unknown | | U.S. |
| | 829 | 33 | 17 | 39 | 30 | 82 | 113 | 43 | RM1182 | Chicken | 1996 | U.S. (Calif.) |
| | 829 | 33 | 17 | 39 | 30 | 82 | 113 | 43 | RM1403 | Chicken | 1997 | Unknown |
| | 829 | 33 | 17 | 39 | 30 | 82 | 113 | 43 | RM2221 | Chicken | 1998 | U.S. |
| | 832 | 33 | 17 | 39 | 30 | 79 | 113 | 43 | RM1169 | Human | | U.S. (Calif.) |
| | 832 | 33 | 17 | 39 | 30 | 79 | 113 | 43 | RM1504 (ATCC 43486) | Unknown | | Unknown |
| | 860 | 33 | 17 | 39 | 30 | 79 | 113 | 47 | RM1840 | Chicken | | Japan |
| | 889 | 33 | 41 | 39 | 30 | 82 | 113 | 47 | RM1166 | Chicken | | Unknown |
| | 889 | 33 | 41 | 39 | 30 | 82 | 113 | 47 | RM1876 (ATCC 43473) | Human | | Belgium |
| | 889 | 33 | 41 | 39 | 30 | 82 | 113 | 47 | RM2230 | Chicken | 1998 | U.S. |
| | 890 | 33 | 36 | 38 | 30 | 82 | 104 | 35 | RM1530 (ATCC 43476) | Sheep | | U.S. |
| | 891 | 33 | 17 | 39 | 30 | 118 | 104 | 64 | RM1531 (ATCC 43478) | Marmoset | | Unknown |
| | 892 | 33 | 17 | 38 | 30 | 115 | 113 | 43 | RM1051 (ATCC 43479) | Human | | Canada |
| | 895 | 82 | 36 | 38 | 30 | 82 | 104 | 35 | RM1532 (ATCC 43482) | Human | | U.S. |
| | 901 | 33 | 41 | 39 | 30 | 79 | 104 | 43 | RM1505 (ATCC 49299) | Unknown | | Unknown |
| | 1016 | 33 | 17 | 38 | 30 | 82 | 118 | 43 | RM3230 (ATCC 51729) | Swine | | Australia |
| | 1016 | 33 | 17 | 38 | 30 | 82 | 118 | 43 | RM3232 | Swine | | Australia |
| | 1017 | 33 | 41 | 39 | 30 | 82 | 104 | 43 | RM1529 | Chicken | 1996 | U.S. |
| | 1049 | 32 | 36 | 39 | 44 | 82 | 104 | 44 | RM1517 | Chicken | 1996 | U.S. |
| | 1050 | 33 | 17 | 39 | 122 | 140 | 113 | 43 | RM1518 | Chicken | 1996 | U.S. |
| | 1050 | 33 | 17 | 39 | 122 | 140 | 113 | 43 | RM1519 | Chicken | 1996 | U.S. |
| | 1051 | 33 | 17 | 39 | 122 | 79 | 209 | 43 | RM1521 | Chicken | 1996 | U.S. |
| | 1053 | 33 | 36 | 39 | 44 | 157 | 104 | 43 | RM1523 | Chicken | 1996 | U.S. |
| | 1054 | 33 | 36 | 39 | 123 | 82 | 104 | 35 | RM1525 | Chicken | 1996 | U.S. |
| | 1055 | 33 | 17 | 39 | 30 | 82 | 104 | 47 | RM1527 | Chicken | 1996 | U.S. |
| | 1055 | 33 | 17 | 39 | 30 | 82 | 104 | 47 | RM1899 | Swine | | U.S. (Tex.) |
| | 1056 | 33 | 36 | 39 | 30 | 82 | 104 | 43 | RM1857 | Human | | Unknown |
| | 1057 | 33 | 17 | 39 | 124 | 78 | 104 | 43 | RM1891 | Chicken | | U.S. (Tex.) |
| | 1058 | 33 | 17 | 39 | 30 | 82 | 104 | 35 | RM1896 | Swine | | U.S. (Tex.) |
| | 1058 | 33 | 17 | 39 | 30 | 82 | 104 | 35 | RM1898 | Swine | | U.S. (Tex.) |
| | 1058 | 33 | 17 | 39 | 30 | 82 | 104 | 35 | RM1900 | Swine | | U.S. (Tex.) |
| | 1058 | 33 | 17 | 39 | 30 | 82 | 104 | 35 | RM1906 | Swine | | U.S. (Tex.) |
| | 1058 | 33 | 17 | 39 | 30 | 82 | 104 | 35 | RM1911 | Swine | | U.S. (Tex.) |
| | 1059 | 33 | 17 | 153 | 30 | 82 | 104 | 35 | RM1897 | Swine | | U.S. (Tex.) |
| | 1061 | 32 | 36 | 39 | 44 | 82 | 104 | 43 | RM1904 | Swine | | U.S. (Tex.) |
| | 1063 | 33 | 41 | 39 | 30 | 140 | 113 | 43 | RM2219 | Chicken | 1998 | U.S. |
| | 1063 | 33 | 41 | 39 | 30 | 140 | 113 | 43 | RM2228 | Chicken | 1998 | U.S. |
| | 1064 | 33 | 41 | 39 | 30 | 82 | 104 | 64 | RM2223 | Chicken | 1998 | U.S. |
| | 1064 | 33 | 41 | 39 | 30 | 82 | 104 | 64 | RM2236 | Chicken | 1998 | U.S. |
| | 1066 | 33 | 41 | 39 | 122 | 82 | 104 | 43 | RM2241 | Chicken | 1998 | U.S. |
| | 1067 | 33 | 41 | 39 | 30 | 140 | 104 | 43 | RM2243 | Chicken | 1998 | U.S. |
| | 1068 | 33 | 17 | 39 | 30 | 78 | 104 | 43 | RM2439 | Manure | 2001 | U.S. (Calif.) |
| | 1069 | 33 | 17 | 38 | 125 | 82 | 118 | 43 | RM3231 (ATCC 51798) | Swine | 1991 | Australia |
| | 1070 | 33 | 17 | 39 | 30 | 79 | 118 | 117 | RM4071 | Swine | | Denmark |
| | 1082 | 33 | 17 | 39 | 30 | 82 | 211 | 85 | RM1178 | Chicken | | U.S. (Calif.) |
| | 1082 | 33 | 17 | 39 | 30 | 82 | 211 | 85 | RM1190 | Chicken | | U.S. (Calif.) |
| | 1082 | 33 | 17 | 39 | 30 | 82 | 211 | 85 | RM2220 | Chicken | 1998 | U.S. |
| ST-1052 complex[b] | 1052 | 53 | 17 | 39 | 44 | 156 | 118 | 35 | RM1524 | Chicken | 1996 | U.S. |
| | 1052 | 53 | 17 | 39 | 44 | 156 | 118 | 35 | RM1522 | Chicken | 1996 | U.S. |
| | 1052 | 53 | 17 | 39 | 44 | 156 | 118 | 35 | RM1526 | Chicken | 1996 | U.S. |
| | 1060 | 53 | 36 | 39 | 44 | 158 | 118 | 35 | RM1901 | Swine | | U.S. (Tex.) |
| Singletons | 898 | 32 | 17 | 42 | 30 | 82 | 104 | 43 | RM1533 (ATCC 43485) | Human | | U.S. |
| | 900 | 32 | 17 | 38 | 30 | 82 | 152 | 35 | RM1515 (ATCC 33559) | Swine | | U.S. |
| | 1062 | 53 | 36 | 38 | 44 | 81 | 118 | 85 | RM1905 | Swine | | U.S. (Tex.) |
| | 1062 | 53 | 36 | 38 | 44 | 81 | 118 | 85 | RM1907 | Swine | | U.S. (Tex.) |
| | 1065 | 103 | 79 | 110 | 30 | 159 | 210 | 164 | RM2225 | Chicken | 1998 | U.S. |

[a] Numbers represent alleles or STs assigned by the *Campylobacter jejuni/coli* MLST database. U.S., United States; Calif., California; Tex., Texas.
[b] No founder ST predicted by eBURST. Lineage named after first identified ST in the complex.

complemented reverse traces indicated that the same secondary peaks were occurring at the same nucleotide positions. Further analysis indicated that these single-nucleotide polymorphisms represented normal allelic variation at these loci. For example, all *C. lari aspA* alleles contain an A or a T at nucleotide 234 and the *C. lari* RM2816 *aspA* traces contain both A and T peaks at that position. This suggested that these "strains," designated as pure cultures, in fact were mixed cultures of two or more strains.

To verify that these strains were mixed, cells were sonicated to break apart potential aggregates, vortexed vigorously, and then dilution plated. Genomic DNA from well-isolated single

TABLE 4. Allele numbers, sequence types, and lineages for *C. lari* isolates ($n = 19$)[a]

| Lineage | ST | Allele | | | | | | | Isolate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *adk* | *atpA* | *glnA* | *glyA* | *pgi* | *pgm* | *tkt* | Name | Source | Yr | Location |
| ST-2 complex[b] | 2 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | RM2099 | Human | | Unknown |
| | 2 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | RM2809 (ATCC 35222) | Unknown | 1982 | Unknown |
| | 6 | 5 | 2 | 1 | 2 | 2 | 1 | 2 | RM2818 (LMG 9253) | Human | 1989 | Unknown |
| | 6 | 5 | 2 | 1 | 2 | 2 | 1 | 2 | RM2820 (LMG 9888) | Seagull | 1986 | Unknown |
| | 6 | 5 | 2 | 1 | 2 | 2 | 1 | 2 | RM2826 (LMG 14338) | Human | 1993 | Belgium |
| Singletons | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | RM1890 (ATCC 43675) | Human | 1985 | Unknown |
| | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | RM2821 (LMG 9889) | Human | 1986 | Unknown |
| | 3 | 2 | 2 | 1 | 2 | 1 | 2 | 3 | RM2100 | Human | | Unknown |
| | 4 | 3 | 3 | 2 | 2 | 3 | 3 | 4 | RM2808 (ATCC 35221) | Unknown | 1980 | UK |
| | 5 | 4 | 4 | 2 | 1 | 2 | 4 | 5 | RM2817 (LMG 9152) | Horse | 1981 | Sweden |
| | 7 | 6 | 5 | 3 | 1 | 3 | 5 | 6 | RM2819 (LMG 9887) | Seagull | | Unknown |
| | 8 | 7 | 1 | 1 | 1 | 1 | 3 | 2 | RM2822 (LMG 9913) | Human | 1987 | Unknown |
| | 9 | 8 | 6 | 1 | 1 | 4 | 1 | 2 | RM2823 (LMG 9914) | Human | 1987 | Unknown |
| | 10 | 9 | 7 | 4 | 3 | 5 | 6 | 7 | RM2824 (LMG 11251) | Unknown | 1991 | Unknown |
| | 11 | 10 | 8 | 5 | 4 | 6 | 7 | 8 | RM2825 (LMG 11760) | Human | 1990 | Canada |
| | 12 | 11 | 9 | 6 | 5 | 7 | 8 | 9 | RM3659 (NCTC 11845) | River water | 1982 | UK |
| | 13 | 12 | 10 | 7 | 6 | 8 | 9 | 10 | RM3660 (NCTC 11928) | River water | 1982 | UK |
| | 14 | 13 | 11 | 8 | 7 | 9 | 10 | 9 | RM3661 (NCTC 11937) | Unknown | 1982 | UK |
| | 15 | 14 | 12 | 9 | 8 | 10 | 11 | 11 | RM4110 (CCUG 22395) | Human | 1986 | France |

[a] Numbers represent alleles or STs assigned by the *C. lari* MLST database. UK, United Kingdom.
[b] No founder ST predicted by eBURST. Lineage named after first identified ST in the complex.

colonies was amplified and sequenced. The resulting allele profiles and STs are shown in Table 7. For *C. coli* strain RM1908 and *C. lari* strain RM2816, two distinct allele profiles are present. Additionally, superimposition of the two forward traces at each locus corresponds accurately with the original "mixed" forward traces, demonstrating that the original RM1908 and RM2816 cultures were mixtures. The distribution of the profiles between the *C. coli* and *C. lari* colonies was approximately 0.3:0.7 (*C. coli*) and 0.5:0.5 (*C. lari*); however, one RM2816 colony remained "mixed" despite sonication and vortexing. Only one allele profile was isolated from *C. upsaliensis* strains RM3949 and RM4048 (Table 7) and *C. helveticus* RM4087 (data not shown), despite two attempts at separation. All traces for the first two strains were unambiguous and corresponded to the primary peaks of the mixed traces at these loci. The second profile (profile II) for each strain was inferred by subtracting the sequence of profile I from the mixed sequence; in most cases, the alleles of the second profile corresponded to alleles identified previously for other strains. The only "mixed" locus in the *C. helveticus* strain RM4087 was *aspA*. Interestingly, the *aspA* allele not obtained after either separation attempt was represented by the dominant profile in the mixture and was identical to the *C. upsaliensis aspA* allele *aspA6*.

**Putative lateral transfer among thermotolerant *Campylobacter* species.** All alleles identified in this study were queried against the *Campylobacter jejuni/coli* MLST database. The *C. coli* alleles at all seven loci were identical to alleles in this database (Table 3). The one exception was *C. coli* RM2225 *aspA*, which was approximately 88% identical to the four other *C. coli aspA* alleles identified in our study but was identical to *C. jejuni aspA103*. Phylogenetic analyses (data not shown) identified two groups of alleles at each locus in the *Campylobacter* MLST database; an additional group (group III) was identified at the *atpA* and *pgm* loci (Table 8). The members of one group (group I or group II) are, on average, about 86% identical to members of the other group. One group of alleles (group II, Table 8) is associated predominantly with *C. coli*. With the exception of RM2225 *aspA*, all of the identified *C. coli* alleles, including the remaining RM2225 alleles, are members of group II. Phenotypic and immunochemical tests indicated that strain RM2225 was a *C. coli* isolate (data not shown); therefore, RM2225 is another example of a *C. coli* strain which contains both *C. jejuni* and *C. coli* MLST loci.

The MLST loci of the other three species characterized in this study have much less similarity to *C. jejuni* MLST loci (78 to 79% nucleotide identity). Therefore, it is noteworthy that *C. lari* RM4110 *pgm11* was identical to the *C. jejuni* allele *pgm110*. The *pgm110*, allele is a member of *pgm* group III (Table 8), a tertiary group of *pgm* alleles identified in the *Campylobacter* MLST database. Group III is only 80 to 81% identical to group I ("*C. jejuni*") alleles and 77 to 79% identical to group II ("*C. coli*") alleles. Significantly, the remaining *C. lari pgm* alleles characterized in this study, such as RM2819 *pgm5*, which is 99.4% identical to *C. jejuni pgm108*, are >94% identical to other group III alleles. These results strongly suggest that the group III *pgm* alleles originated in *C. lari*. Additionally, they also suggest that the typed strains in the MLST database containing these alleles may have resulted from lateral transfer events between *C. jejuni* and *C. lari*.

Based on the above results, it was possible that MLST might reveal lateral transfer events between the four non-*C. jejuni* species. Therefore, pairwise BLAST combinations of all alleles were performed. Two groups of *C. helveticus pgm* alleles were identified: those with approximately 86% identity to *C. upsaliensis pgm* alleles, similar to the nucleotide identity at the other six loci, and *pgm* alleles with approximately 96% nucleotide identity to *C. upsaliensis pgm* alleles. Thus, these results suggest that lateral transfer events may have occurred between

TABLE 5. Allele numbers, sequence types, and lineages for *C. upsaliensis* isolates ($n = 76$)[a]

| Lineage | ST | Allele | | | | | | | Isolate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *adk* | *aspA* | *atpA* | *glnA* | *glyA* | *pgi* | *tkt* | Name | Source | Yr | Location |
| ST-16 complex[b] | 16 | 7 | 11 | 1 | 9 | 9 | 12 | 1 | RM3810 | Cat | 2003 | U.S. (Calif.) |
| | 65 | 17 | 11 | 1 | 9 | 9 | 12 | 1 | RM4417 | Dog | 2004 | U.S. (Calif.) |
| ST-35 complex[b] | 35 | 13 | 17 | 1 | 12 | 1 | 12 | 1 | RM4058 | Human | 1992 | S. Africa |
| | 62 | 1 | 17 | 1 | 26 | 1 | 12 | 1 | RM4410 | Dog | 2004 | U.S. (Calif.) |
| ST-42 complex | 6 | 4 | 5 | 6 | 6 | 5 | 6 | 6 | RM3776 | Human | 1996 | S. Africa |
| | 7 | 4 | 6 | 7 | 7 | 5 | 6 | 6 | RM3777 | Human | 1997 | S. Africa |
| | 8 | 4 | 6 | 7 | 5 | 5 | 7 | 6 | RM3778 | Human | 1997 | S. Africa |
| | 9 | 4 | 7 | 5 | 5 | 6 | 6 | 6 | RM3779 | Human | 2002 | S. Africa |
| | 10 | 4 | 8 | 5 | 7 | 5 | 6 | 6 | RM3780 | Human | 2002 | S. Africa |
| | 11 | 4 | 9 | 5 | 6 | 5 | 8 | 6 | RM3781 | Human | 2002 | S. Africa |
| | 12 | 4 | 6 | 5 | 5 | 5 | 6 | 6 | RM3783 | Human | 2002 | S. Africa |
| | 12 | 4 | 6 | 5 | 5 | 5 | 6 | 6 | RM3786 | Human | 2003 | S. Africa |
| | 12 | 4 | 6 | 5 | 5 | 5 | 6 | 6 | RM4040 | Human | 2001 | S. Africa |
| | 12 | 4 | 6 | 5 | 5 | 5 | 6 | 6 | RM4046 | Human | 2001 | S. Africa |
| | 12 | 4 | 6 | 5 | 5 | 5 | 6 | 6 | RM4047 | Human | 2001 | S. Africa |
| | 29 | 4 | 19 | 5 | 5 | 5 | 6 | 15 | RM4039 | Human | 2000 | S. Africa |
| | 30 | 4 | 19 | 5 | 5 | 5 | 6 | 6 | RM4042 | Human | 2001 | S. Africa |
| | 30 | 4 | 19 | 5 | 5 | 5 | 6 | 6 | RM4044 | Human | 2001 | S. Africa |
| | 30 | 4 | 19 | 5 | 5 | 5 | 6 | 6 | RM4061 | Human | 2001 | S. Africa |
| | 36 | 4 | 5 | 5 | 6 | 5 | 6 | 6 | RM4059 | Human | 1992 | S. Africa |
| | 37 | 4 | 6 | 5 | 5 | 6 | 6 | 6 | RM4062 | Human | 2001 | S. Africa |
| | 38 | 4 | 6 | 7 | 20 | 5 | 6 | 6 | RM4063 | Human | 2001 | S. Africa |
| | 40 | 14 | 4 | 5 | 5 | 5 | 6 | 6 | RM4065 | Human | 2001 | S. Africa |
| | 42 | 4 | 6 | 7 | 5 | 5 | 6 | 6 | RM4068 | Human | 2003 | S. Africa |
| ST-45 complex[b] | 45 | 16 | 25 | 14 | 23 | 19 | 10 | 21 | RM4134 (CCUG 23017) | Human | 1988 | France |
| | 54 | 20 | 28 | 14 | 21 | 19 | 21 | 21 | RM4251 (LMG 9222) | Human | 1987 | Belgium |
| | 57 | 16 | 25 | 14 | 21 | 19 | 21 | 21 | RM4254 (LMG 9234) | Human | 1987 | Belgium |
| ST-50 complex | 50 | 19 | 26 | 16 | 21 | 23 | 19 | 25 | RM4245 (LMG 9108) | Human | 1986 | Belgium |
| | 50 | 19 | 26 | 16 | 21 | 23 | 19 | 25 | RM4248 (LMG 9125) | Human | 1986 | Belgium |
| | 53 | 19 | 27 | 16 | 21 | 23 | 19 | 25 | RM4250 (LMG 9140) | Human | 1986 | Belgium |
| | 60 | 19 | 26 | 16 | 21 | 23 | 24 | 25 | RM4257 (LMG 9265) | Human | 1987 | Belgium |
| | 61 | 19 | 27 | 16 | 21 | 23 | 19 | 28 | RM4258 (LMG 9269) | Human | 1987 | Belgium |
| ST-64 complex[b] | 64 | 1 | 33 | 1 | 13 | 28 | 12 | 12 | RM4414 | Dog | 2004 | U.S. (Calif.) |
| | 64 | 1 | 33 | 1 | 13 | 28 | 12 | 12 | RM4415 | Dog | 2004 | U.S. (Calif.) |
| | 66 | 1 | 33 | 1 | 13 | 28 | 12 | 4 | RM4418 | Dog | 2004 | U.S. (Calif.) |
| | 66 | 1 | 33 | 1 | 13 | 28 | 12 | 4 | RM4419 | Dog | 2004 | U.S. (Calif.) |
| Singletons | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | RM1488 (ATCC 49815) | Human | | Canada |
| | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | RM2092 | Human | | Unknown |
| | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | RM2093 | Human | | U.S. |
| | 4 | 3 | 3 | 4 | 4 | 1 | 4 | 4 | RM2094 | Human | | U.S. |
| | 5 | 4 | 4 | 5 | 5 | 4 | 5 | 5 | RM3195 | Human | 1994 | S. Africa |
| | 13 | 4 | 10 | 8 | 7 | 7 | 9 | 7 | RM3784 | Human | 2002 | S. Africa |
| | 14 | 5 | 6 | 7 | 5 | 8 | 10 | 6 | RM3785 | Human | 2003 | S. Africa |
| | 15 | 6 | 3 | 1 | 8 | 3 | 11 | 8 | RM3808 | Dog | 2003 | U.S. (Calif.) |
| | 17 | 6 | 12 | 1 | 10 | 1 | 13 | 9 | RM3812 | Dog | 2003 | U.S. (Calif.) |
| | 18 | 8 | 13 | 1 | 11 | 1 | 14 | 10 | RM3937 | Human | 1998 | U.S. (Calif.) |
| | 19 | 9 | 14 | 1 | 12 | 10 | 12 | 4 | **RM3939** | Human | 1998 | U.S. (Calif.) |
| | 20 | 6 | 15 | 1 | 13 | 11 | 1 | 11 | RM3940 | Human | 1998 | U.S. (Calif.) |
| | 21 | 0 | 16 | 9 | 14 | 1 | 12 | 12 | RM3941 | Human | 1998 | U.S. (Calif.) |
| | 22 | 10 | 3 | 9 | 10 | 12 | 12 | 12 | RM3942 | Human | 1998 | U.S. (Calif.) |
| | 23 | 10 | 17 | 10 | 3 | 13 | 15 | 4 | RM3943 | Human | 1998 | U.S. (Calif.) |
| | 24 | 1 | 17 | 10 | 13 | 14 | 12 | 13 | RM3944 | Human | 1998 | U.S. (Calif.) |
| | 25 | 6 | 18 | 11 | 15 | 1 | 16 | 1 | **RM3945** | Dog | 1998 | U.S. (Calif.) |
| | 25 | 6 | 18 | 11 | 15 | 1 | 16 | 1 | **RM3946** | Dog | 1998 | U.S. (Calif.) |
| | 26 | 10 | 14 | 10 | 16 | 1 | 14 | 8 | RM3947 | Dog | 1998 | U.S. (Calif.) |
| | 27 | 1 | 17 | 1 | 17 | 13 | 4 | 14 | **RM3948** | Dog | 1998 | U.S. (Calif.) |
| | 28 | 1 | 13 | 1 | 18 | 11 | 1 | 11 | RM3950 | Dog | 1998 | U.S. (Calif.) |
| | 31 | 4 | 20 | 5 | 5 | 7 | 6 | 16 | RM4043 | Human | 2001 | S. Africa |
| | 32 | 11 | 21 | 5 | 19 | 5 | 6 | 6 | RM4049 | Human | 2002 | S. Africa |
| | 33 | 12 | 22 | 12 | 19 | 15 | 7 | 17 | RM4051 | Human | 2003 | S. Africa |
| | 34 | 5 | 7 | 5 | 6 | 16 | 17 | 6 | RM4055 | Human | 1992 | S. Africa |
| | 39 | 5 | 8 | 13 | 5 | 5 | 6 | 18 | RM4064 | Human | 2001 | S. Africa |
| | 41 | 4 | 23 | 7 | 21 | 17 | 6 | 19 | RM4066 | Human | 2003 | S. Africa |

*Continued on following page*

TABLE 5—*Continued*

| Lineage | ST | Allele | | | | | | | Isolate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *adk* | *aspA* | *atpA* | *glnA* | *glyA* | *pgi* | *tkt* | Name | Source | Yr | Location |
| | 43 | 15 | 24 | 12 | 22 | 18 | 9 | 20 | RM4123 (CCUG 19559) | Human | 1986 | UK |
| | 44 | 6 | 15 | 1 | 1 | 1 | 13 | 1 | RM4133 (CCUG 14913) | Dog | 1980 | Sweden |
| | 46 | 13 | 18 | 4 | 24 | 20 | 12 | 22 | RM4135 (CCUG 20818) | Human | | U.S. |
| | 47 | 17 | 15 | 11 | 1 | 1 | 14 | 9 | RM4136 (CCUG 33890) | Dog/cat | 1995 | Sweden |
| | 48 | 18 | 17 | 15 | 13 | 21 | 3 | 23 | RM4137 | Unknown | | Scotland |
| | 49 | 16 | 4 | 14 | 21 | 22 | 18 | 24 | RM4244 (LMG 9104) | Human | 1986 | Belgium |
| | 51 | 16 | 25 | 17 | 7 | 17 | 18 | 21 | RM4246 (LMG 9114) | Human | 1986 | Belgium |
| | 52 | 7 | 12 | 1 | 9 | 24 | 20 | 1 | RM4249 (LMG 9129) | Human | 1986 | Belgium |
| | 55 | 1 | 29 | 18 | 25 | 25 | 22 | 26 | RM4252 (LMG 9226) | Human | 1987 | Belgium |
| | 56 | 17 | 30 | 11 | 1 | 26 | 16 | 1 | RM4253 (LMG 9230) | Human | 1987 | Belgium |
| | 58 | 21 | 31 | 19 | 7 | 27 | 9 | 7 | RM4255 (LMG 9240) | Human | 1987 | Belgium |
| | 59 | 16 | 32 | 20 | 7 | 17 | 23 | 27 | RM4256 (LMG 9261) | Human | 1987 | Belgium |
| | 63 | 1 | 17 | 1 | 1 | 28 | 12 | 14 | RM4411 | Dog | 2004 | U.S. (Calif.) |

[a] Boldface indicates *C. upsaliensis* isolates originating from the same household. Numbers represent alleles or STs assigned by the *C. upsaliensis* MLST database. U.S., United States; Calif., California; S. Africa, South Africa; UK, United Kingdom.
[b] No founder ST predicted by eBURST. Lineage named after first identified ST in the complex.

*C. upsaliensis* and *C. helveticus* at the *pgm* locus. However, no other significant identities were observed at any other loci.

## DISCUSSION

A multilocus sequence typing method was developed previously for *C. jejuni* (12). Although this method has been used successfully to characterize *C. jejuni* strains and identify clonal lineages within the species (6, 11, 13, 43, 60), it cannot characterize strains from other, clinically relevant *Campylobacter* species (e.g., *C. coli* and *C. upsaliensis*), nor can it address interspecies genetic exchange within the genus. Therefore, an expanded MLST typing system was developed, encompassing four additional species: *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. Where possible, the same housekeeping genes used in the *C. jejuni* MLST method were incorporated into the expanded method. This was feasible for *C. coli* and *C. helveticus*; however, it was necessary to substitute *adk* and *pgi* for *aspA* and *gltA* in *C. lari* and for *gltA* and *pgm* in *C. upsaliensis*.

A large number of alleles at each locus were identified in *C. lari* and *C. upsaliensis* relative to the number of strains analyzed (Table 2). Despite the small number of *C. helveticus* strains, several alleles were also identified at each locus in this species (Table 2); based on the number of identified alleles at each locus, the potential number of allele combinations (i.e., *C. helveticus* STs) is predicted to be at least 56,000. In contrast, relatively few alleles were identified in *C. coli*. Considering that a similar number of *C. coli* and *C. upsaliensis* isolates were characterized, approximately four times more alleles were identified in *C. upsaliensis*, suggesting that *C. upsaliensis* is more genotypically diverse than *C. coli*. Nonetheless, the majority of *C. coli* strains (38/56; 68%) contained unique STs, indicating that these seven loci are sufficient to type this species. In fact, the majority of strains typed in this study contained unique STs.

In the absence of codon usage bias, the rate of synonymous base substitution (which does not change the amino acid) in genes should equal the neutral substitution rate; nonsynonymous base substitutions (which change the amino acid) would be caused and maintained presumably by positive selection. Therefore, the ratio of nonsynonymous to synonymous base substitutions ($d_n/d_s$) is an indicator of potential positive selection; such positive selection might make a gene unsuitable for MLST. By calculating the $d_n/d_s$ ratio, Dingle et al. (12) demonstrated that the MLST loci in their typing scheme were not subject to positive selection. The $d_n/d_s$ values for *C. coli* (0 to 0.173), *C. lari* (0 to 0.047), and *C. upsaliensis* (0.008 to 0.097) (Table 2) are consistent with those described previously for *C. jejuni* (0.028 to 0.059 [12] and 0.008 to 0.093 [11]). The values

TABLE 6. Allele numbers, sequence types, and lineages for *C. helveticus* isolates ($n = 8$)[a]

| Lineage | ST | Allele | | | | | | | Isolate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *aspA* | *atpA* | *glnA* | *gltA* | *glyA* | *pgm* | *tkt* | Name | Source | Yr | Location |
| ST-1 complex[b] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | RM3228 (ATCC 51209) | Cat | | Switzerland |
| | 5 | 4 | 1 | 1 | 1 | 5 | 1 | 1 | RM4139 (CCUG 34042) | Cat | 1995 | Sweden |
| Singletons | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | RM3229 (ATCC 51210) | Cat | | Switzerland |
| | 3 | 2 | 1 | 2 | 1 | 3 | 3 | 2 | RM3807 | Cat | 2003 | U. S. (Calif.) |
| | 4 | 3 | 2 | 1 | 3 | 4 | 2 | 2 | RM4088 (CCUG 34016) | Cat | 1995 | Sweden |
| | 6 | 5 | 3 | 3 | 3 | 6 | 4 | 2 | RM4140 (CCUG 30563) | Cat | 1991 | Switzerland |
| | 7 | 6 | 4 | 4 | 1 | 7 | 2 | 1 | RM4141 (CCUG 30564) | Cat | 1991 | Switzerland |
| | 8 | 7 | 5 | 5 | 4 | 8 | 5 | 2 | RM4142 (CCUG 30683) | Cat | 1992 | Switzerland |

[a] Numbers represent alleles or STs assigned by the *C. helveticus* MLST database. U.S., United States; Calif., California.
[b] No founder ST predicted by eBURST. Lineage named after first identified ST in the complex.

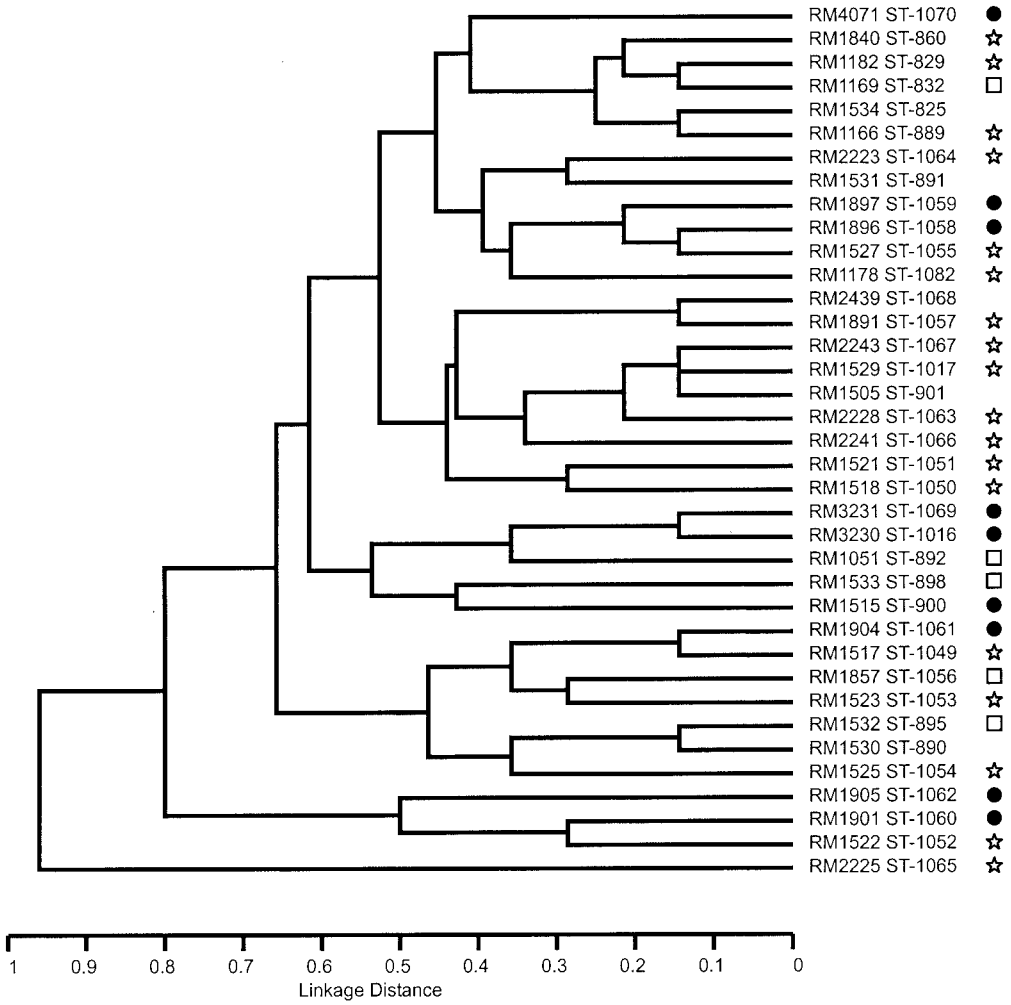


FIG. 1. UPGMA clustering of *C. coli* strains. Chicken strains are annotated with stars; swine strains are annotated with filled circles; human strains are annotated with open squares.

for *C. helveticus* are higher for some loci (Table 2) but are less than 1 in all cases. These results indicate that, as with *C. jejuni*, the MLST loci of the other four species are not subject to positive selection. The ability of the primer sets to amplify and sequence strains of each species from a variety of clinical and environmental sources, sufficient genotypic variation at each of the seven MLST loci, and the absence of positive selection all demonstrate that the expanded MLST method is a suitable typing scheme. Additionally, although the primer sets described by Dingle et al. (12) are sufficient to type *C. jejuni* isolates, the primer sets described in this method can be used also to amplify and sequence *C. jejuni* alleles (Table 1).

For *C. coli* and *C. lari*, there was no correlation between sequence or allele type and strain source (e.g., clinical versus environmental). A correlation with source could not be made with *C. helveticus* either since all of the strains were feline isolates. However, six clonal complexes were identified for *C. upsaliensis* (Table 5): the ST-42 complex, comprised exclusively of clinical isolates from South Africa; the ST-45 and ST-50 complexes, comprised exclusively of clinical isolates from Belgium and France; and the ST-16, ST-35, and ST-64 complexes, comprised primarily of isolates from household pets. UPGMA analysis of the *C. upsaliensis* allele profiles identified two distinct genogroups (Fig. 2). With one exception (RM4123, isolated in the United Kingdom), genogroup II is comprised of the South African, Belgian, and French isolates.

Several groups of strains with identical STs were found in *C. coli*, *C. lari*, and *C. upsaliensis* (e.g., $ST_C$-1058, $ST_L$-6, and $ST_U$-12; Tables 3 to 5). For some of these groups, e.g., $ST_C$-1058, the strains were all isolated from one location during the same year. However, the strains in many groups were isolated over the course of several years ($ST_L$-6, 7 years) or from widely separated geographical locations ($ST_C$-889). Additional typing will be required to determine if these groups represent prevalent *Campylobacter* strains. *C. coli* strains RM3230 and RM3232 have identical STs. Both strains are swine isolates from Australia. Investigation of the strain background for RM3232 indicated that this isolate was most likely RM3230, further illustrating the power of MLST to identify identical strains.

The ability to source track isolates from both sporadic and outbreak cases is a goal for most prokaryotic typing systems. The large number of alleles and STs in *C. lari*, *C. upsaliensis*,

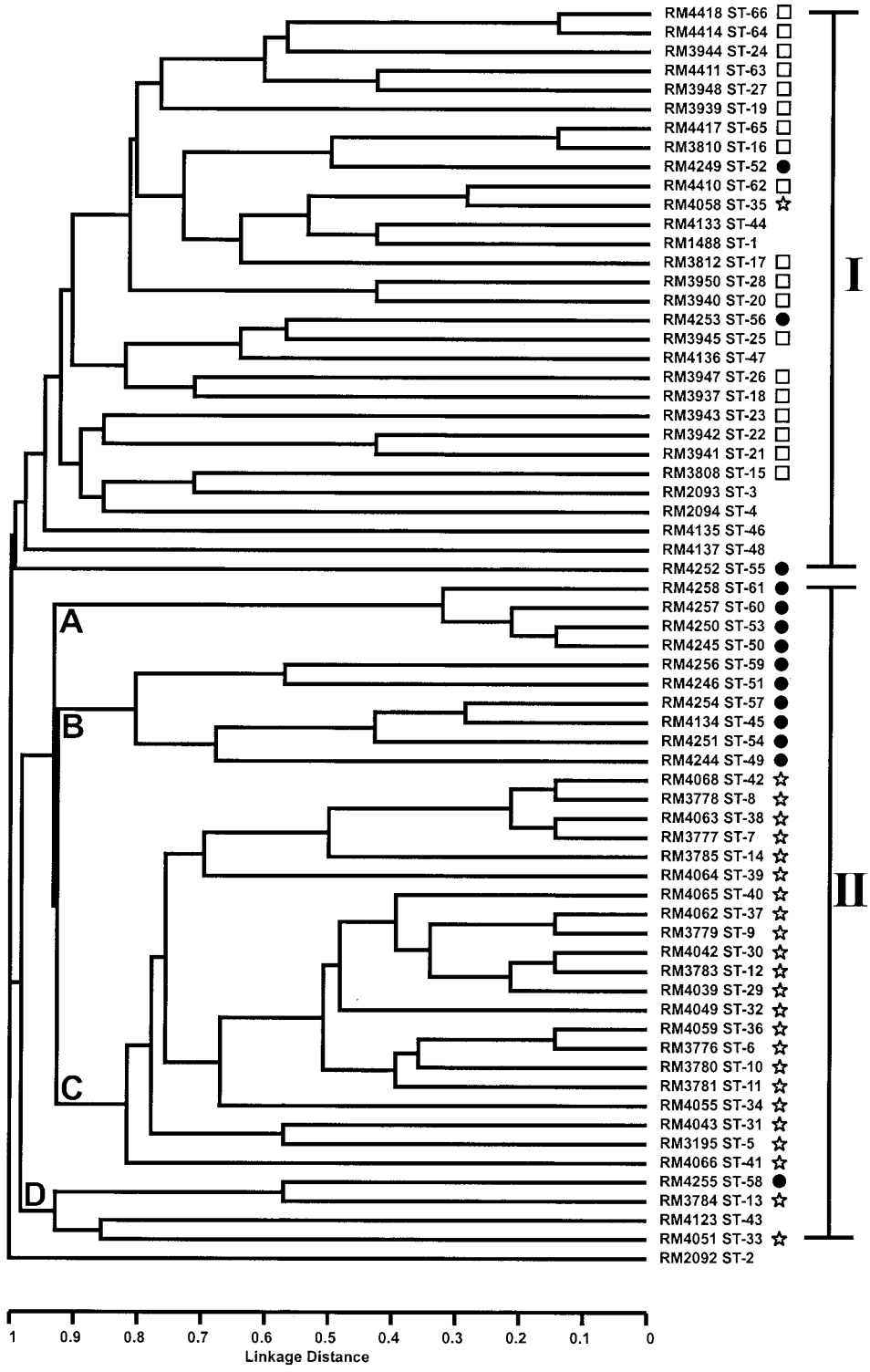


FIG. 2. UPGMA clustering of *C. upsaliensis* strains. South African *C. upsaliensis* strains are annotated with stars; strains from Belgium or France are annotated with filled circles; strains from California are annotated with open squares.

and *C. helveticus* would suggest that source tracking may be possible with MLST, although the potential of source tracking in *C. lari* and *C. helveticus* cannot be adequately addressed in this study due to the small set of typed isolates. All of the *C. upsaliensis* isolates were obtained from patients with campylobacteriosis or from domestic pets. While this precludes tracking these clinical isolates back to a food or water source, it does illustrate the potential use of MLST to investigate the zoonotic

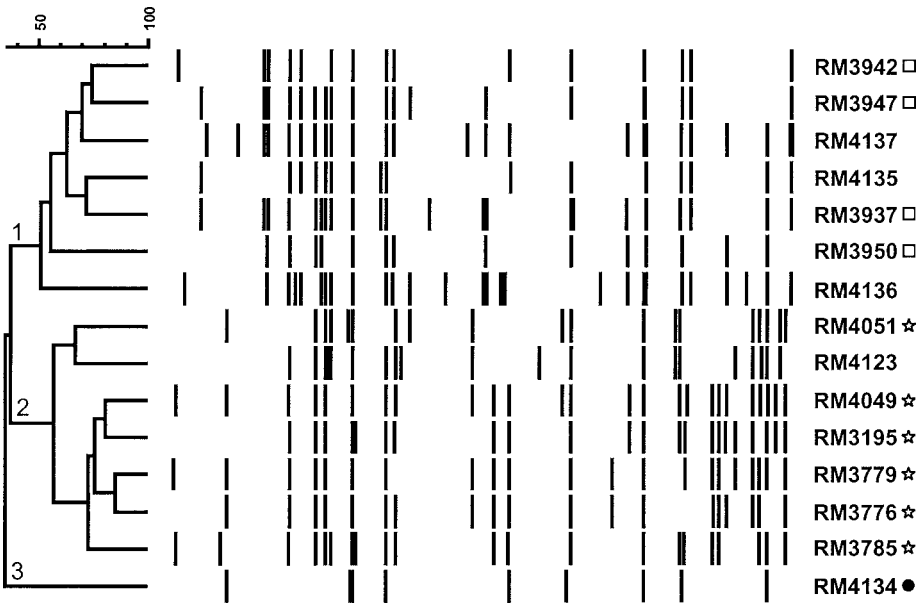


FIG. 3. AFLP phenogram for a representative set of *C. upsaliensis* strains. South African *C. upsaliensis* strains are annotated with stars; a strain from Belgium or France is annotated with a filled circle; strains from California are annotated with open squares. Phenogram derived from numerical analysis of AFLP profiles based on the Dice coefficient and UPGMA clustering. Reconstructed band profiles are displayed to the left of strain designations. The scale bar represents percent similarities between strains as determined by the coefficients used.

transmission of *C. upsaliensis*. As described above, *C. upsaliensis* is commonly isolated from domestic dogs and cats; therefore, handling of these animals, especially by children, is one possible mode of transmission for this organism. Labarca et al. (37) found that *C. upsaliensis* isolates recovered from a patient with gastroenteritis and dogs living in the same household had different pulsed-field gel electrophoresis (PFGE) patterns; similar results were obtained after typing these isolates by MLST ($ST_U$-19, $ST_U$-25, and $ST_U$-27; Table 5). However, the stool specimens from the pets were obtained several months after *C. upsaliensis* was isolated from the owner; therefore, it is unclear, due to the lag between the isolation of the two sample sets, if the gastroenteritis was a result of zoonotic transmission. In contrast to *C. upsaliensis*, no correlation in *C. coli* between ST and either location/date of isolation or source was found, suggesting that source tracking might not be possible in *C. coli* with our MLST loci. Another possibility is that the number of *C. coli* strains typed in this study was, again, inadequate to address the potential of source tracking by MLST. This appears to be the case. In a related study, over 500 chicken, turkey, swine, and cattle *C. coli* isolates were typed using the MLST method described here. Several alleles were found to be predominantly associated with either chicken or swine isolates (W. G. Miller, unpublished data), suggesting that source tracking by MLST may be possible in *C. coli*.

It is noteworthy that all *C. upsaliensis* genogroup II strains (with the possible exception of RM4123) were isolated using a filtration protocol with no antibiotic selection (27, 44), referred to as the Cape Town Protocol filtration method (41), whereas most of the isolates in *C. upsaliensis* genogroup I were isolated on agar amended with cefoperazone or cephalothin. In contrast to many strains in genogroup I, strain RM3195 (isolated

TABLE 7. Allele profiles and sequence types for the separated alleles of strains RM1908, RM2816, RM3949, and RM4048[a]

| Name | Type | *adk* | *aspA* | *atpA* | *glnA* | *gltA* | *glyA* | *pgi* | *pgm* | *tkt* | ST | Source | Yr | Location |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. coli* RM1908 | I | NT | 53 | 36 | 38 | 44 | 81 | NT | 118 | 85 | 1062 | Swine | Unk. | USA (Tex.) |
| | II | NT | 33 | 36 | 38 | 30 | 81 | NT | 118 | 35 | Unk. | Swine | Unk. | USA (Tex.) |
| *C. lari* RM2816 (LMG 8844) | I | 15 | NT | 13 | 10 | NT | 9 | 11 | 12 | 12 | 16 | Seawater | 1989 | UK |
| | II | 7 | NT | 1 | 1 | NT | 1 | 1 | 3 | 2 | 8 | Seawater | 1989 | UK |
| *C. upsaliensis* RM3949 | I | 1 | 13 | 1 | 18 | NT | 11 | 1 | NT | 11 | 28 | Canine | 1998 | USA (Calif.) |
| | II[b] | 6 | 17 | 1 | 16 | NT | 28 | 1 | NT | 9 | 62 | Canine | 1998 | USA (Calif.) |
| *C. upsaliensis* RM4048 | I | 4 | 6 | 7 | 7 | NT | 5 | 6 | NT | 6 | 7 | Human | 1997 | S. Africa |
| | II[b] | 22 | 21 | 5 | 19 | NT | 5 | 6 | NT | 6 | 63 | Human | 1997 | S. Africa |

[a] I and II signify each sequence type in the mixed culture. NT, not tested; Unk., unknown; USA, United States; Tex., Texas; UK, United Kingdom; Calif., California; S. Africa, South Africa.

[b] Strains RM3949-II and RM4048-II were not recovered after sonication; alleles and sequence types are inferred by subtraction of the RM3949-I and RM4048-I alleles from the mixtures.

TABLE 8. Divergent alleles in the *Campylobacter* MLST database[a]

| Locus | Group | Alleles[b] | % Nucleotide identity to other *C. jejuni* alleles |
|---|---|---|---|
| *aspA* | II | **32**, **33**, 49, 50, 51, 52, **53**, 54, 58, 60, 66, 72, 78, 81, **82**, 86, 88, 89, 90 | 87–89 |
| *atpA* | II | **17**, 28, **36**, 37, 38, **41**, 42, 67, 68, 73, 74, 75, 76, **79** | 85–88 (85–88 between groups I and II) |
| | III | 56 | 85–88 |
| *glnA* | II | 37, **38**, **39**, **42**, 47, 49, 66–68, 86–88, 90, 91, 92, 104, 108, **110**, 116, 124, **153** | 87–89 |
| *gltA* | II | **30**, 32, 36–38, **44**, 46, 65–67, 69, 81, 83, 86–88, 103, **122**, **123**, **124**, **125** | 87–89 |
| *glyA* | II | 32, 41, 59, 75, 76, **78**, **79**, **81**, **82**, 102, 113, 114, **115**, 116, **118**, 124, 139, **140**, **156**, **157**, **158**, **159** | 82–85 |
| *pgm* | II | 48, 65, 71, 93, **104**, 111, 112, **113**, **118**, 143, **152**, 160–166, 188, 189, **209**, **210**, **211** | 84–87 (77–79 between groups I and II) |
| | III | 100, 108, 109, *110* | 80–81 |
| *tkt* | II | **35**, **43**, **44**, **47**, 56, 63, **64**, 65, 71, 72, 77, 84, **85**, **117**, 119, 122, 126–131, 138, **164** | 85–87 |

[a] Divergent alleles were identified by CLUSTALW and BLASTN analysis of alleles in the *Campylobacter jejuni*/*coli* MLST database. Total alleles at the *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt* loci are 111, 91, 153, 125, 159, 211, and 165, respectively.
[b] Boldface alleles were found in *C. coli* isolates in this study. Italic alleles were found in *C. lari* isolates in this study.

by filtration) is highly sensitive to cefoperazone (21). This suggests that the strains in genogroup II have increased cefoperazone sensitivity and that genogroups I and II are both genotypically and phenotypically distinct. The existence of two groups of *C. upsaliensis* strains has been confirmed also by additional genotypic and phenotypic data (C. K Fagerquist et al., unpublished data; R. E Mandrell et al., unpublished data). The existence of a *C. upsaliensis* subpopulation, more likely to be isolated by non-antibiotic selection methods (e.g., filtration), suggests that additional subpopulations of other *Campylobacter* species (e.g., *C. jejuni* and *C. coli*) remain undetected due to the almost universal use of antibiotic selection media. The possibility that subpopulations of strains are not being isolated has important implications for the identification of virulence factors, the sources of antibiotic resistance, and accurate epidemiology.

In addition to MLST, other typing methods, such as PFGE and AFLP analyses, have been described for *Campylobacter* (50). Although PFGE is used commonly as a typing method in *Campylobacter* (http://www.cdc.gov/pulsenet/), the results are more prone to subjective interpretation and lab-to-lab variation than results from other, sequence-based methods. To compare our MLST method to existing typing methods, we typed a representative subset of *C. upsaliensis* strains by AFLP and compared the interstrain relationships derived from the two methods. The correlation between interstrain relationships inferred by MLST and AFLP analyses has been noted previously with a comparison of methods previously described for *C. jejuni* (63). We noted a similar correlation between our MLST and AFLP results for *C. upsaliensis*. All strains studied gave unique types in both methods. Furthermore, the cluster analyses of data derived from each method detected the same degree of relatively close, or also distant but discernible, interstrain relationships among strains. Strains assigned to the same ST complex also shared a high level of AFLP profile similarity. The results were epidemiologically significant, with strains in AFLP cluster 1 dominated by strains from the United States and those in cluster 2 dominated by South African isolates. These data validate both MLST and AFLP as complementary genotyping methods that have applicability in evaluating both epidemiological and genealogical relationships of *Campylobacter* spp. and demonstrate the relationship between the genotype and phylotype of strains.

Two additional benefits of the expanded MLST method were revealed in this study, i.e., detection of presumed "pure" cultures containing two strains of the same species and lateral transfer of DNA between *Campylobacter* species. We had reported previously that well-isolated single colonies could contain two strains, observed after plating mixtures of two fluorescence-tagged *C. jejuni* strains (47). Additionally, mixed cultures containing multiple strains representing different *Campylobacter* species have been reported previously (16, 56, 67). Obviously, mixed cultures, containing strains of different species, can be detected readily by other, less labor-intensive methods, such as PCR. However, detecting mixtures of the same species is very difficult without prior knowledge of the genotypes or phenotypes of the strains composing the mixture, as the differences between the strains are likely to be minor; however, in some instances, mixed cultures of the same species can be detected when the component strains have noticeably different colony morphologies (38). The ability to identify the presence of mixed cultures is important since it has obvious implications in both outbreak source tracking and monitoring of antibiotic resistance, as well as basic strain characterization. Therefore, it is very important to ensure that pure cultures have been obtained for characterization. Five mixed cultures, representing all four species, were clearly detected by the MLST system developed in this study. Four of the strains were environmental isolates, obtained from animals or seawater (Table 7). Environmental samples, as opposed to clinical samples, might be expected to contain multiple strains of the same species; therefore, special care should be taken when purifying strains from such samples. It is also relevant that one well-isolated, single colony of the *C. lari* strain RM2816 remained mixed, even after sonication and vortexing, illustrating the difficulty of separating mixed cultures. Although pure cultures

of *C. upsaliensis* were obtained from "strains" RM3949 and RM4048, the secondary strain in each mixture could not be isolated. One explanation is that the secondary strain represented a minor proportion of the mixture. However, it was determined previously that mixtures in a ratio of greater than 4:1 cannot be detected by DNA sequencing, since the secondary peaks become indistinguishable from the background (data not shown). Therefore, it is unlikely that MLST would identify a mixture in which the primary strain was in >8-fold excess. A more probable explanation is that the secondary strains did not survive the sonication process or that they grew much more slowly on the BAB medium. Two strains, annotated originally as *C. upsaliensis* and *C. helveticus*, were determined in this study to be *C. jejuni* (data not shown). Since a number of well-characterized tests for *C. jejuni* exist and would have been used on these strains, it is also possible that the original cultures were mixed and eventually outgrown, during multiple passages, by minor *C. jejuni* subpopulations in the mixtures. Similar results with other *Campylobacter* mixtures have been seen previously (M. Englen, personal communication).

Multilocus sequence typing can also detect putative lateral transfer events between species. Expansion of the *C. jejuni* MLST to five *Campylobacter* species presents a unique opportunity to monitor genetic exchange between multiple species within the genus. Detection of such events between *C. jejuni* and *C. coli* has been reported previously (45, 63). In fact, 55 STs in the *Campylobacter* MLST database are composed of both "*C. jejuni*" and "*C. coli*" alleles. In 22 of these STs, the sole "*C. coli*" allele is *atpA17*, an allele strongly associated with the *C. jejuni* ST-61 complex. The potential association of this allele with *C. coli* has been noted previously (11, 12, 45, 63). It is likely that all of the group II alleles (Table 8) present in the database are derived from *C. coli*, due to the identity or near-identity to *C. coli* alleles or *C. coli* alleles characterized previously or in this study. Interestingly, *C. lari* alleles are also present apparently in the MLST database (group III: $pgm100_J$, $pgm108_J$, $pgm109_J$, and $pgm110_J$); the *C. lari pgm* allele $pgm11_L$ is identical to $pgm110_J$. The $atpA56_J$ allele is also a group III allele (Table 8). BLAST analysis indicates that it is most related (89% identity) to the *atpA* alleles from the urease-positive *C. lari* strains RM3659, RM3660, and RM3661 (data not shown); however, the low identity suggests that this allele may belong to either a divergent *C. lari* genogroup or another species related to *C. lari*. The presence of only two *C. lari*-related loci in the *C. jejuni* MLST database likely precludes the possibility of these alleles being characterized via accidental typing of one or more *C. lari* isolates and suggests rather that these alleles are present in *C. jejuni* as a result of lateral transfer. Other than these group II and III alleles, no other potential lateral transfer events were detected in this study, with the exception of putative *C. upsaliensis pgm* (and possibly *aspA*) alleles in *C. helveticus*.

The small number of putative lateral transfer events detected in this study may reflect the relatively small sample size for each of the five species as well as the expected low frequency for such events. Additional lateral transfer events are likely to be identified as more strains are typed by this method. The small number of identified putative lateral transfer events may also reflect local synteny around each of the seven MLST loci. Presumably, the absence of conserved flanking genes would decrease the likelihood of recombination at each locus. Characterization of synteny between *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* strains is facilitated by the existence of closed genomes for the first three species and a draft genome for the fourth (21; W. G. Miller et al., unpublished data). Of course, an important qualification is that there might be significant differences in synteny between the sequenced strains and other strains from the same species, especially species with a high degree of inherent variation, such as *C. lari*. Nevertheless, the gene order around the seven MLST loci is very similar in the sequenced *C. coli* strain RM2228 and either of the two sequenced *C. jejuni* genomes (NCTC 11168 and RM1221). Conversely, the regions around the MLST loci in *C. lari* strain RM2100 and *C. upsaliensis* strain RM3195 are not very syntenic with similar regions in either *C. jejuni* or *C. coli*; in most cases, the breakpoint in gene order is either immediately adjacent to or within one gene of the MLST gene. This difference in synteny among species may explain the large number of *C. coli* alleles and the relatively small number of *C. lari* and *C. upsaliensis* alleles present in the *Campylobacter* MLST database. However, a few non-*C. jejuni*/*coli atpA* and *pgm* alleles described in this study have been identified in the *C. jejuni* MLST database. *atpA* is the middle gene in the *atpF'FHAGDC* locus. Unlike the other six MLST loci, this extended locus is highly conserved among the four sequenced species; therefore, one might expect a higher frequency of allelic exchange at the *atpA* locus. Although the gene order downstream of *pgm* in *C. jejuni* and the urease-negative *C. lari* strain RM2100 is well conserved, the gene order upstream is not. However, the group III *pgm* alleles are most similar to the *pgm* alleles from the urease-positive *C. lari* strains. Therefore, it is possible that the upstream gene order at the *pgm* locus is more conserved between urease-positive *C. lari* strains and *C. jejuni*. Similar differences in synteny have been observed in *C. lari* at other loci (W. G. Miller, unpublished data). These results suggest also that genetic exchange of MLST genes between two species might be confined to small subsets of strains within those species.

This study describes an expanded multilocus sequence typing method for five *Campylobacter* species. While the expanded method will efficiently characterize these pathogenic, emerging campylobacters, the five-species MLST method will also prove useful in identifying both lateral transfer between *Campylobacter* species and mixed cultures. Allele and profile data from this study are available online (http://pubmlst.org/campylobacter/, http://pubmlst.org/clari/, http://pubmlst.org/cupsaliensis/, and http://pubmlst.org/chelveticus/). Therefore, as more strains, both clinical and environmental, from these species become available, they can be compared readily to existing members of the database. Finally, while this method characterizes mainly the thermotolerant *Campylobacter* species (i.e., *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*), preliminary data suggest that this expanded method can be expanded further still. The *atpA* and *glyA* primer sets were used to amplify successfully genomic DNA from eight additional *Campylobacter* species (*C. fetus*, *C. hyointestinalis*, *C. sputorum*, *C. mucosalis*, *C. concisus*, *C. curvus*, *C. showae*, and *C. lanienae*). Noteworthy is the identification of a mixed *C. mucosalis* culture and the presence of a *C. jejuni glyA* allele (*glyA27*) in *C. showae*, suggesting that an MLST method encompassing all members of the genus can

provide useful insights into *Campylobacter* biology and evolution.

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